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13. ABSTRACT (Maximum 200 words) We proposed to develop nipple fluid-based test(s) for early breast cancer detection. These non-invasive breast nipple fluid studies could complement mammography, particularly for women under age 50. Our studies in breast nipple fluid have examined 2 tumor biomarkers, carcinoembryonic antigen and prostate-specific antigen (CEA and PSA). In clinically cancer-free women, CEA titers vary widely in 281 breast nipple fluid samples. The median CEA is 1,057 ng/ml, which is more than 200-fold higher than normal CEA serum levels. Likewise, PSA median level in nipple fluids is 49 ng/ml, when serum PSA is virtually 0 in women. High CEA and PSA levels in nipple fluid were new and unexpected findings. Our IDEA study was to determine whether CEA and PSA levels are biomarkers for breast cancer. Nipple fluid CEA and PSA titers from 45 women with untreated breast cancer and 60 with DCIS, LCIS or ADH were compared with titers for the 281 cancer-free women. CEAs in fluids from cancerous breast are significantly higher than CEA levels in normal breasts ($p < 0.01$). No differences were found between CEAs in precancerous breasts and normal breasts. PSA levels were comparable in all 3 subgroups. Studies are needed to identify other biomarkers in NAFs with high positive predictive values for cancer.				
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FOREWORD

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INTRODUCTION: Breast cancer is the most common cancer in American women. Cure rates for breast cancer are highly correlated with early disease stage at initial diagnosis. Recent decline in breast cancer mortality rates in the U.S. and elsewhere is likely due to the benefits of both early detection and more effective treatments. Mammography is an established screening modality that has been repeatedly shown to reduce breast cancer mortality in women age 50 and over. Clinical breast exam and breast self-examination are additional methods of early detection, but have low sensitivity.

In this IDEA project, we are seeking to complement mammography and physical exams with novel breast cancer detection methods based on nipple fluid analyses. We have examined whether carcinoembryonic antigen (CEA) and prostate specific antigen (PSA) in breast nipple fluid can be used as biomarkers of early breast cancer. Nipple fluid can be obtained in approximately 50 percent of all American women, or 50 million potential beneficiaries of a validated test if found. Nipple fluid CEA and PSA studies offer several attractive features. Firstly, the methods of specimen collection, developed nearly 50 years ago, are safe and non-invasive. Also, standard laboratory assays for CEA and PSA are available and inexpensive. Although CEA and PSA in nipple fluid have not been examined previously, elevated CEA titers in spontaneous pathologic breast nipple discharges are reportedly predictive of breast cancer. Other new tumor markers, when identified, can also be sought in the cellular and liquid fractions of breast fluid.

A year ago, we reported that cancer-free women have high levels of CEA in nipple fluid (median CEA, 1,087 ng/ml). We have now examined both CEA and PSA titers in nipple fluids from pre-operative cancer-bearing breasts, and compared results with fluids from normal breasts. Nipple fluid analyses can be extended in future studies to identify new candidate biomarkers for cancer. Our studies have also yielded new insights into sample procurement and analytical strategies for future NAF research.

BODY:

Experimental Methods. Nearly 50 years ago Papanicolaou, the developer of the Pap smear for cervical cancer, pioneered the use of a breast pump to obtain nipple fluid for cytologic analysis for breast cancer (1). A limitation to nipple fluid analyses has been the small quantity of the material. Approximately 10-100ul of nipple fluid is obtainable from 30-60% of American women. The fluid is more readily obtained from women under age 50, for whom mammography may provide benefit (2-4). Breast cancers have been diagnosed by nipple fluid cytology in anecdotal published reports (1, 5, 6). Thus, there are presently 50 million potential candidates for nipple fluid exams in the U.S., if validated early cancer detection tests were to become available (7, 8).

Petrakis has analyzed nipple fluid collected from nearly 50,000 women in recent decades (7, 8). In a prospective study of 2,701 women with nearly 30,000 person-years of follow-up, subjects whose nipple fluid showed cytologic atypia had an increased risk of subsequent breast cancer (relative risk=4.9; 95%CI, 1.7-13.9) (9). However, cytologic diagnosis is limited by the presence of only 100 breast epithelial cells in the typical nipple aspirate specimen, and the Petrakis results are unconfirmed (5, 8, 9). Investigators have also found that the liquid fraction of nipple fluid contains high levels of various steroid hormones, proteins, lipids, and growth factors (8, 10, 11) but not tumor biomarkers indicative of early breast cancers.

Hypothesis. We propose that CEA and PSA in breast nipple fluids are early markers of breast cancer. These 2 tumor biomarkers have been widely studied in peripheral blood using standard laboratory assays. CEA is an oncofetal antigen that is detected in 50-90% of primary breast tumors using immunohistochemical techniques. CEA is often elevated in serum of patients with metastatic breast cancer, and is widely used to monitor response to cancer treatment (12). Recent studies in Japan of abnormal spontaneous breast discharge (not nipple fluid collected by suction) have found elevated CEA

levels among women whose workup revealed a previously unrecognized breast cancer. Inaji et al. reported using a different assay that showed CEA levels in spontaneous abnormal nipple discharge exceeded 600 ng/ml in 6 of 7 women with nonpalpable early breast cancers (tumor cells were later shown to express CEA), and 0 of 23 with non-cancerous breast discharges (13). Three additional reports from Japan have confirmed the finding (14-16). In one, high nipple discharge CEA titers had a sensitivity of 76% and specificity of 79% for marking the presence of breast cancer (16). In the past, prostate-specific antigen (PSA) was thought to be produced only in male reproductive organs, and thus, absent in women (17). Serum PSA levels are low (up to 4 ng/ml) in healthy men, and elevated serum PSA levels are clinically used as a marker for prostate cancer. Serum PSA is undetectable in healthy women. However, recent reports have identified high PSA levels in both extracts of female breast cancers and in cell culture media of PSA-positive breast tumor lines (17-21). Our primary objective of this project is to initiate the multistep process of examining nipple fluid CEA and PSA as candidate biomarkers for the neoplasm, and to solve methodologic problems associated with sample collection and data analyses.

Procedures. We developed procedures to collect nipple fluids and risk factor data by questionnaire from women with pre-operative breast lesions, and cancer-free controls. Using standard assays, PSA and CEA will be assayed to determine whether these established tumor markers are significantly higher in nipple fluids from cancer cases as compared with controls. CEA and PSA are being compared in pre-treatment breast cancer patients, women with DCIS, LCIS or ADH, and women without cancer (controls). Sample size is limited by the cost of identifying and enrolling untreated breast cancer patients in the short interval between cancer diagnosis and surgery. During this period, patients are anxious and we take the time to be supportive and sensitive to their distress. We have obtained IRB approval to collect and analyze breast nipple fluid samples from women through 5 Boston hospitals that register 2000 breast cancer cases annually. These are Dana-Farber Cancer Institute, Massachusetts General Hospital, Brigham and Women's Hospital, Beth Israel Hospital and Faulkner Hospital. Samples are processed and analyzed under supervision of the PI in the Molecular Diagnostics Laboratory of Dana Farber Cancer Institute, using methods pre-tested in our preliminary studies.

With signed consent from an eligible woman, the breast nipple is gently cleansed. She is asked to gently compress her breast. The suction cup is placed over the nipple, and suction is gently applied for 5-10 seconds using a 20 ml syringe. The procedure is less uncomfortable and less intrusive than breast compression for mammograms. If a droplet appears, the fluid is collected into a microcentrifuge tube and the process is repeated on the opposite breast. The residual moisture on the nipple is spread onto a slide and stored for future cytologic studies. The fluid is centrifuged, and the cell pellet and supernatant are separately aliquoted for storage at -70C. No major complications from the procedure have been encountered in 1000 collection attempts. The procedure itself takes about 5 minutes and is well tolerated. Finding potential participants, explaining the procedure, obtaining informed consent, and collection of basic risk factor data take more than 30 minutes. In the last year, we have been faced with increasing demands for higher patient throughput, particularly in mammography centers, as participating hospitals increasingly struggle with budget deficits. Near to end of the project, one mammography center was forced to withdraw from participation because our study prolonged patients' visits. Unfortunately, nipple fluid collection for research purposes, when performed with sensitivity and respect for the participant, is a labor intensive process. The nipple fluid samples were examined for CEA using the commercial immunoenzymometric assay kit, AIA-PACK CEA (Tosoh Medics, Foster City, CA).

Results. We have examined CEA and PSA titers in nipple fluids from 386 women. Samples were re-tested and showed highly reproducible results. Right and left breast fluid samples for cancer-free subjects consistently yielded comparable CEA titers (Spearman rank correlation=0.78, $p<0.01$). The median CEA in normal breasts was 1,057 ng/ml. The 45 specimens from women with pre-operative breast cancers

showed a median titer of 1,565 ng/ml, whereas 60 samples from women who had DCIS, LCIS and ADH have immediate levels (1,396 ng/ml). Samples were also tested by Western blot using a mouse anti-human monoclonal antibody (IgG1; Piece, Rockford, Illinois) along with controls (purified human CEA protein; Calbiochem Novabiochem, San Diego, CA). These nipple fluids contained the predicted 180 kD CEA glycoprotein. CEA in suctioned nipple fluid had never been reported previously (22).

Table 1 summarizes CEA and albumin-adjusted CEAs. When the CEA levels were adjusted for albumin levels in the nipple fluids, the statistically significant increase among preoperative breast cancer cases persisted. However, the CEA levels were comparable in fluids from breasts with precancerous lesions and normal breasts. For PSA, crude and albumin-adjusted levels were comparable in breasts containing cancer or precancerous lesions, as compared with tumor free breasts. These findings suggest that nipple fluid CEA may be useful as an assay for early breast cancer, whereas PSA in nipple fluid does not distinguish between normal and cancer.

Table 1. CEA and albumin-adjusted CEA levels in nipple aspirate fluid (NAF), by breast tumor status at sample collection.

Diagnosis at NAF collection	No. of subjects	Median (mean) of biomarker titers	Crude OR (p value)	Adjusted OR (p value)
<u>CEA</u>				
Preoperative breast cancer	45 (44)	1565 (3474)	3.4 (0.0070)	4.0 (0.0003)
DCIS, LCIS, ADH	60 (56)	1396 (5509)	1.4 (0.2238)	1.5 (0.1966)
Tumor-free (controls)	281 (273)	1057 (1831)	1.0	1.0
<u>CEA/ALB</u>				
Preoperative breast cancer	45(44)	44 (82)	2.0 (0.0319)	2.1 (0.0279)
DCIS, LCIS, ADH	60 (56)	25 (182)	0.9 (0.7517)	1.0 (0.9184)
Tumor-free (controls)	281 (273)	29 (56)	1.0	1.0

In a small number of study subjects, we were able to discern the ductules from which the nipple fluids were obtained. The droplets of fluid from different ductules were collected and analyzed separately. These droplets from different ducts often had different color and viscosity, and their CEA and PSA levels were also different. These findings established for the first time differences in titers of biomarkers depending on the ductule from which the fluid was obtained. Thus, but lack of test sensitivity of the two assays may be due in part to blockage of certain ducts by the cancerous mass.

In the meantime, we have explored other novel assays. In collaboration with Dr. Jerry Shay at MD Anderson Hospital, we have begun to look at telomerase levels in nipple fluids. However, available assays did not have adequate sensitivity to detect telomerase in our small quantities of material. In a second collaboration with Matritech, a biotechnology firm focusing on nuclear matrix proteins (23, 24), NAF samples showed differing patterns of concentration and distribution of diverse proteins. Analysis was made of a breast cancer-associated nuclear matrix protein developed by Matritech. In 28 specimens, this nuclear matrix protein was found to be expressed in different levels among nipple fluid specimens, but levels do not correlate with clinical status. A third project in collaboration with Dr.

Susan Love will focus on the cellular components of NAFs. By suction, only 100 epithelial cells are collected. A new method has now been developed that yields up to 10,000 cells. This advance in sample collection creates new opportunities to apply new molecular genetic methods with the promise of being able to detect a single cancer cell.

KEY RESEARCH ACCOMPLISHMENTS

- consented 1314 women to answer risk factor questionnaire and consent to nipple fluid collection
- successfully collected nipple fluids from 449 women
- analyzed nipple fluid CEA and PSA, as well as nipple fluid albumin levels in 386 study subjects
- demonstrated significantly elevated levels CEA in breasts of 45 women with preoperative breast cancer, as compared with nipple fluids from 281 women without breast tumors.
- in women with pre-cancerous breast lesions, CEA levels were not elevated significantly when compared with control samples
- PSA levels in nipple fluids of women with breast cancer were not significantly different from corresponding PSA levels in women with normal breasts
- CEA and PSA levels can differ among ductules draining to a single nipple. Thus, a nipple fluid sample represents the composite of potential biomarker titers from multiple breast ducts.

REPORTABLE OUTCOMES

- Two publications will result from this work (see page 8, Publications and Abstracts)
- We have collected a repository of nipple fluids.
- Dr. Lenka Foretova, the post-doc on the project, has received a faculty appointment in her native country (Czech Republic), and is continuing our collaboration.

CONCLUSIONS: We are studying small quantities of nipple fluids, and we need to find assays most useful for identifying breast cancer. A new and promising development is the ability to collect many more breast epithelial cells for studies of known breast cancer-associated genes in nipple fluids. The method may prove useful in finding cancer-associated mutations in affected breasts, so early detection can be improved.

PUBLICATIONS AND ABSTRACTS RESULTING FROM THIS WORK

(see Appendix for reprints)

Foretova L, Garber JE, Sadowsky NL, Verselis SJ, Joseph DM, Andrade AFF, Gudrais PG, Fairclough

D, Li FP. Carcinoembryonic antigen in breast nipple aspirate fluid. *Cancer Epi Bio Preven* 1998; 7:195-198.

Zhao Y, Verselis S, Gudrais P, Foretova, L, Klar H, Li FP. Nipple fluid CEA and PSA in breast cancer pateints. *In preparation*

PERSONNEL

Frederick Li	Principal Investigator	6/97-5/99
Judy Garber	Co-Investigator	6/97-5/99
Arlene Kantor	Epidemiologist	10/97-5/99
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Miriam Hawkes	Research Nurse	3/98-1/99
Christine Henault	Data Manager	10/97-5/99
Zhiming Mai	Data Manager	2/99-5/99
Katie Nicholls	Data Manager	3/99-5/99

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Carcinoembryonic Antigen in Breast Nipple Aspirate Fluid¹

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Abstract

New diagnostic tools are needed to complement mammography and physical examinations for early detection of breast cancer, particularly among younger women. We evaluated the tumor biomarker, carcinoembryonic antigen (CEA), in 215 nipple aspirate fluid (NAF) samples collected from one or both breasts of 147 women, ages 27–87 years. Most subjects were recruited at the time of mammography examination. The 215 nipple fluid CEAs range from undetectable levels to 8400 ng/ml (median, 1100 ng/ml). Normal serum CEA levels are less than 6 ng/ml. There are no significant differences between the CEAs in fluid from normal breasts (112 samples) and breasts with various histories of tumors (total, 103 samples). Analyses for determinants of CEAs in fluids from normal breasts show higher levels among current smokers ($P = 0.03$) and marginal elevations among nulliparous women ($P = 0.07$). CEAs in these samples are not correlated with age, menopausal status, current hormone use, prior breastfeeding, or family history of breast cancer. Follow-up studies of these women and comparisons of CEAs in fluids from normal and cancer-containing breasts will help clarify whether this biomarker is useful for risk assessment or early cancer detection.

Introduction

One woman in nine in the United States eventually develops breast cancer (1). Mammograms and physical examinations are the standard methods for early breast cancer detection. Routine mammography screening has been shown to reduce breast cancer mortality among women ages 50 years and over and probably among women in their 40s (2–4). However, accuracy

of mammography is lower among young women, and additional early detection methods are needed (5).

For several decades, cells and the supernatants from NAFs³ have been examined for evidence of early breast cancer and biomarkers of high cancer risk. Early studies focused on cytological examination of epithelial cells in NAF for dysplasia and carcinoma (6–12). Other reports have described various biochemical constituents of NAF, including immunoglobulins, lipids, cholesterol, fatty acids, lactose, hormones, and growth factors (9, 13–17). Recently, we and others reported high levels of PSA in NAF (18, 19).

CEA was identified in 1965 as the first human cancer-associated antigen and serological tumor biomarker (20). CEA is a secreted protein, and elevated serum CEAs are found in patients with diverse forms of advanced cancers, particularly carcinomas of the colon, breast, and lung (21). CEA is elevated in serum of 40–50% of patients with metastatic breast cancer, and is used both in initial tumor staging and monitoring of response to treatment (22). CEA is detectable immunohistochemically in breast cancer cells, whereas most of the normal and benign tumor tissues stain weakly or not at all with anti-CEA antibodies (23). CEA has been detected in foamy macrophages and intraluminal material of nonneoplastic lobules and ducts adjacent to the CEA-positive cancerous tumors (23, 24). The physiological role of CEA in breast nipple fluid and the clinical significance of high NAF concentrations have not been studied previously. This study describes the range of CEA levels in NAFs and several correlates of CEA elevations.

Materials and Methods

Subjects. Participants were recruited between 1993 and 1996 in ambulatory clinics and mammography suites of four Boston hospitals (Dana-Farber Cancer Institute, Faulkner Hospital, Beth-Israel Hospital, and Brigham and Women's Hospital). Excluded from the study were lactating women and those with bleeding tendencies, scarred nipples, local infections, or spontaneous bloody nipple discharge. Informed consent was obtained to perform breast nipple aspiration on 474 women who responded to a brief questionnaire on demographic characteristics, breast diseases, and breast cancer risk factors. Their mammography and breast biopsy results were obtained from a review of available medical records.

Specimen Collection Procedures. NAFs were collected using techniques described previously (7). In brief, nipples were cleansed with alcohol swabs to remove cellular debris. The large majority of samples were collected by placing over the nipple a small plastic suction cup attached to a 20-ml syringe, and applying suction for several seconds. Recently, we found that NAFs can also be successfully collected from women who can manually express fluid from their breasts. Droplets of

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³ The abbreviations used are: NAF, nipple aspirate fluid; CEA, carcinoembryonic antigen; PSA, prostate-specific antigen.

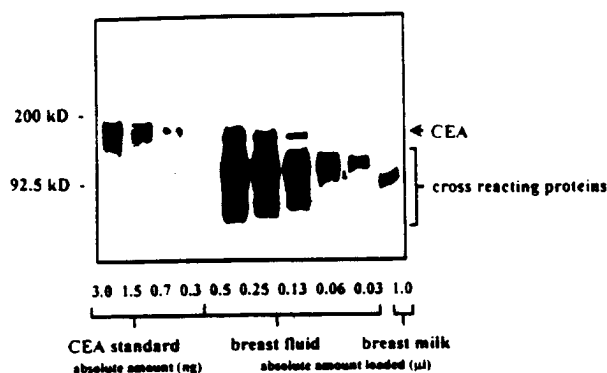


Fig. 1. Western blot of CEA in serially diluted CEA standard (four left lanes), breast fluid (next five lanes), and breast milk sample (right lane). Both the M_r 180,000 CEA band and the cross-reacting species of lower molecular weight are detected by the mouse anti-human CEA monoclonal antibody.

nipple fluid were collected into microcentrifuge tubes. The volume of NAFs varied from 0–280 μ l, usually 5–20 μ l. Nipple suction was well tolerated by nearly all subjects and was stopped if substantial discomfort was encountered. No serious complications occurred. As control specimens, 20 breast milk samples were obtained from the Regional Milk Bank of The Medical Center of Central Massachusetts.

Laboratory Methods. NAFs were transported on ice to the laboratory within 8 h after collection. NAFs were usually viscous and were diluted up to 10-fold in 1 \times PBS and centrifuged. The supernatant was aliquoted for storage at -70°C . Quantitative CEA assays were performed using the commercial immunoenzymometric assay kit AIA-PACK CEA (Tosoh Medics, Foster City, CA), which has a lower detection limit of 0.1 ng/ml. Due to high CEA levels in most NAFs, samples were diluted (1:200) before analysis with CEA Sample Diluting Solution (Tosoh Medics). Reproducibility of the assay was examined in 21 samples by repeating the NAF dilution and CEA analysis steps. The replicate error accounts for 1% of the total CEA variation. To demonstrate detection of the M_r 180,000 CEA glycoprotein, Western blots were performed using purified human CEA (Calbiochem Novabiochem, San Diego, CA) as a standard protein and a mouse monoclonal anti-human CEA antibody (IgG1 subclass; Pierce, Rockland, IL). Western blots of 20 NAFs showed the expected M_r 180,000 CEA glycoprotein along with CEA cross-reacting species of lower molecular weight (Fig. 1). In addition, CEA titers were normalized to albumin levels, which were determined in duplicate by colorimetric reaction with Bromocresol green (Sigma Diagnostics, St. Louis, MO). Albumin levels vary more widely in NAFs (range, 2–100 mg/ml; median, 29 mg/ml) than in serum (39–50 mg/ml).

Data Analysis. The 215 NAFs were considered the units of analysis unless otherwise noted. Based on a review of questionnaires and available medical records, breasts were classified as normal when there was no history of cancer, precancerous lesions (ductal carcinoma *in situ*, lobular carcinoma *in situ*, or atypical duct hyperplasia), or nonneoplastic conditions (Table 1). Median, \log_{10} of the mean, and range of CEA levels were analyzed for all samples and subsets of NAFs. The variability of replicate CEA determination was estimated from the variance components of a general linear model. The correlation of CEA levels between the right and left breasts was estimated using the Pearson coefficient. Associations of CEA levels with

Table 1 CEA levels in nipple aspirate fluid (NAF) samples, by history of breast neoplasia

Clinical status at NAF collection	No. of samples	CEA (ng/ml)	
		Median ^a	Range
Preoperative breast invasive carcinoma ^b	4	2000	800–6400
Postoperative breast neoplasia (carcinoma, DCIS, LCIS, ADH) ^c	16	1400	<100–3770
Preoperative nonneoplastic conditions ^d	43	1500	<100–6630
Postoperative nonneoplastic conditions ^d	40	800	<100–8440
Normal breast ^e	112	1100	<100–7530
Total	215	1100	<100–8400

^a No statistically significant differences between CEA from normal breasts, as compared with breasts with various abnormalities ($P > 0.05$ for each of the four disease categories when compared with normals).

^b NAF collected before surgical excision of cancer.

^c NAF collected after excision of cancer. DCIS (ductal carcinoma in situ), LCIS (lobular carcinoma in situ), ADH (atypical ductal hyperplasia).

^d Non-neoplastic conditions include fibroadenoma, fibrocystic disease, cyst, sclerosing adenosis, papilloma.

^e No history of cancer or other listed lesions.

factors such as age, parity, lactation history, menstrual status, smoking history, family history, and diagnoses were estimated using Kendall's Tau-b and associated hypothesis tests (25). To account for multiple collections and bilateral NAF samples, statistical inferences regarding the effect of such factors as smoking and parity were adjusted for multiple measurements in applicable subjects by using the average CEA level per subject (26).

Results

NAFs were obtained from one or both breasts of 147 of the 474 women (31%). Donors were 27–87 years of age (median, 44 years), and all but five were Caucasian. Similar to other reports, our rates of successful collection declined with age: 47% before age 40, 38% at ages 40–49, 22% at ages 50–59, and 14% thereafter (27). Ninety-seven of the 147 women (66%) were premenopausal, 42 (29%) had a first-degree relative with breast cancer, 29 (20%) were active smokers, and 20 (14%) were taking hormones for contraception or menopause (8–10). Eight women were receiving cancer therapy or were within 6 months of completing treatments. One hundred seven (73%) had one or more completed pregnancies, and 70 (65%) of these mothers breastfed their infants.

Nipple fluids were obtained from both breasts of 56 women, and 9 provided serial samples. A total of 215 NAFs were collected from 112 breasts without any history of tumors (normals), 4 from breasts with newly diagnosed breast cancers, 16 from breasts after excision of various *in situ* and invasive neoplasms, and 83 from pre- or postoperative breasts with benign diseases (fibrocystic disease, cyst, fibroadenoma, papilloma, and sclerosing adenosis).

CEAs in the 215 NAFs range from undetectable levels (<100 ng/ml after accounting for dilution) to 8400 ng/ml (median, 1100 ng/ml). CEA levels in nipple fluid exceed normal serum CEA levels (0–6 ng/ml) by approximately 200-fold. CEAs normalized to albumin in nipple fluid range from undetectable to 1700 ng CEA/mg albumin (median, 40 ng CEA/mg albumin; data not shown). The CEA:albumin ratios for NAFs also exceed the corresponding serum ratios by 200-fold. CEA

Table 2 Effect of parity and current smoking on CEA levels in nipple aspirate collected from normal breasts

Subject characteristics	No. of subjects ^a	CEA (ng/ml)	
		Median	Range
Current smokers ^b	18	1600	170-6600
Non-smokers	69	800	<100-7500
Parous ^b	68	1000	<100-5800
Nulliparous	24	1400	<100-7500

^a Subjects were 93 women who produced NAF from normal breasts. Average CEA levels were used for subjects who provided paired or multiple samples. Missing are data on smoking by six women and parity of one woman.

^b $P = 0.03$ for smoking; $P = 0.07$ for parity.

levels are highly correlated in 18 paired samples collected from normal breasts ($r = 0.61$, $P = 0.01$) and 20 paired samples from one normal breast and a breast treated previously for benign disease ($r = 0.68$, $P = 0.001$).

Unlike NAF, 15 of 20 undiluted breast milk samples tested have no detectable CEA (<0.1 ng/ml), and the other 5 breast milks have CEA levels of 0.1-2.0 ng/ml (data not shown). Western blots of breast milk do not exhibit the M_r 180,000 CEA glycoprotein but show the same CEA cross-reacting species detected in Western blots of NAFs (Fig. 1).

Table 1 shows CEA levels by clinical status of the breast at NAF collection (preoperative breast cancer, postoperative breast neoplasia, pre- and postoperative benign masses, and normals). CEA levels in 112 NAFs from normal breasts range from undetectable to 7500 ng/ml (median, 1100 ng/ml). Among the 103 breasts with various histories of tumors, NAFs from 4 newly diagnosed cancer-bearing breasts have the highest CEA levels (median, 2000 ng/ml), but the excess is small and non-significant. In addition, no significant CEA elevations are found in NAFs from breasts that had been treated for benign tumors, *in situ* and invasive carcinomas, as well as breasts containing masses that eventually proved benign.

The 112 NAFs from normal breasts of 93 women were examined for associations between CEA levels and donor characteristics. The 18 current smokers have significantly higher CEA levels when compared with nonsmokers ($P = 0.03$; Table 2). In addition, CEAs were moderately decreased among 68 parous women when compared with the nulliparous ($P = 0.07$). CEA levels showed no association with subjects' age at study, menopausal status, hormone use at the time of fluid collection, prior breastfeeding, or family history of breast or ovarian cancer in a first-degree relative (data not shown).

Discussion

In addition to developing cytological diagnosis for carcinoma of the cervix, Papanicolaou studied other body fluids for cytological evidence of cancer (6). In 1958, he described the collection of NAFs with a suction device and reported finding atypical cells in 27 of 45 NAFs collected from patients bearing breast cancers (6). In a prospective study of 2701 women, Wrensch *et al.* (12) report the association between cytological atypia in NAF cells and breast cancer development up to 15 years later. NAF cytology has not proven useful in cancer diagnosis, however, because of its low predictive value. Major technical limitations are low NAF volumes, paucity of breast epithelial cells, and difficulties in distinguishing cancer from dysplastic cells (12).

NAF supernatants have been examined for a variety of biochemical constituents. Some display much higher levels in

NAF as compared with serum, whereas others do not. We have recently described high PSA levels (median, 55 ng/ml) in NAFs that do not correlate with personal or family history of breast cancer (18). Sauter *et al.* (19) also found elevated PSA levels in NAFs from cancer-free subjects, which vastly exceed PSAs in fluids collected from their mastectomy specimens containing invasive cancer. The discordant results might be due to differences in the collection and assay techniques. Among other NAF constituents, levels of cholesterol, estrogen, and gross cystic disease fluid protein are at least 10-fold higher than subjects' corresponding blood levels (9, 13-16, 28). Total protein and IgA levels are elevated to lesser extents, whereas albumin, IgG, and IgM levels are lower in NAFs for unknown reasons (9). Because breasts are secretory glands, concentrations of various NAF constituents might be determined by uptake from blood, synthesis and degradation by breast epithelial cells, secretion into breast ductules and resorption, and processing by macrophages and other cells in NAFs (9). These mechanisms might also affect levels of CEA and other tumor biomarkers in NAF.

We examined CEA levels in NAFs to determine whether this tumor biomarker might be useful for breast cancer detection. Study subjects differed with regard to their past history of breast tumors, clinical status at NAF collection, and risk factors for breast cancer. Median CEA level in NAFs of normal breasts (1100 ng/ml) is 200-fold higher than the normal serum CEA (<6 ng/ml); CEA concentrations of 1100 ng/ml in serum are virtually diagnostic of extensive cancer and poor prognosis (22). CEA levels are high in NAFs from normal breasts and concordant in bilateral samples of normal breast pairs. We found no significant differences between nipple fluid CEA levels for normal breasts and breasts with various breast tumors. However, only four NAFs from cancer-containing breasts were available for study because samples were collected primarily in mammography units. CEAs for these cancerous breasts are slightly higher than levels for normal breasts, but the difference is small and nonsignificant.

Our search for determinants of nipple fluid CEAs for normal breasts revealed higher titers in active smokers. Smoking moderately increases serum CEA levels by unknown mechanisms and may have a similar effect in breast fluid (29). Smoking is an important risk factor for many cancers, but its role in breast cancer development remains uncertain (30, 31). CEA levels in NAF do not rise with age, as reported with serum CEA (29). In addition, CEA levels tend to be slightly higher in nulliparous women than in parous women.

Several Japanese studies evaluated CEA levels in spontaneous pathological nipple discharge, which differs from our NAFs collected by suction or manual breast compression. Using a semiquantitative dot-immunobinding assay (32, 33), CEAs from spontaneous nipple discharges were elevated (>400 ng/ml in their assays) in 74% of one series of patients with palpable breast cancer and 76% of patients with nonpalpable breast cancer but in only 24% of patients without cancer. The reported sensitivity, specificity, and accuracy of the CEA nipple discharge were 76, 79, and 78%, respectively (33). The differences in CEAs between the Japanese studies and our series may be due to differences in ethnicity, assay techniques, and pathogenesis of the abnormal discharges. The explanation can be examined by international exchange of samples for duplicate analyses. The Japanese finding of higher CEA in discharge samples from cancerous breasts, if confirmed, may be useful for cancer detection in the few women with spontaneous nipple discharge.

In this study, we found high CEA levels in NAF and identified several determinants of elevated titers. Follow-up of

our series might show whether high CEAs are predictive for future breast cancer development. In addition, a case-control study comparing CEA levels in nipple fluids from normal and cancerous breasts can determine the usefulness of this biomarker for early cancer detection. If nipple fluid CEAs have acceptable predictive values, this low-cost, noninvasive test might be an adjunct to mammography for early cancer detection.

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